Recent evidence for oncogenesis by insertion mutagenesis and gene activation

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Summary

Putative cellular oncogenes have recently been identified by their homology with known viral oncogenes and by their capacity to transform the behaviour of cultured cells. Retroviruses lacking oncogenes may induce tumours by altering or activating such cellular oncogenes. Analysis of such tumours reveals novel mechanisms for modulating expression of eukaryotic genes, identifies new candidate oncogenes, and suggests possible mechanisms for oncogenesis by non-viral agents.

Introduction

For many years, tumour virologists paid lip service to the possibility that the chromosomal sites at which viral DNA integrates might have a determining influence upon the neoplastic process. Until recently, this notion has had few adherents, for several good reasons. (i) Insertion of viral genomes into host DNA seemed most likely to affect host genes by disrupting them; it is difficult to envisage a cancer arising from the inactivation of one gene in a diploid genome. (ii) Although more promising consequences of viral insertions were plausible (e.g., gene activation, creation of a hybrid gene, or inactivation of a gene at a locus for which the host was heterozygous), such speculations were unsupported by experimental observations. (iii) The integrated (proviral) forms of DNA and RNA tumour viruses appeared to be inserted randomly in

the host genome, reducing the likelihood of introducing viral DNA at the presumably rare positions conducive to oncogenesis. (iv) Mounting evidence for transforming genes (oncogenes) carried by many DNA and RNA tumour viruses seemed to obviate the need for more complicated and unsubstantiated mechanisms of viral oncogenesis.

Events of the past couple of years have refocused attention upon insertional mechanisms for viral oncogenesis and have provided some specific experimental reagents with which to pursue the idea that many types of cancer of unknown cause—not only certain virus-induced cancers—might have their roots in the disordered expression of host genes. My aim in this brief review is to provide the nonspecialist with an understanding of the experimental findings that have contributed to the current viewpoint.

Two overlapping areas of study are central to the ensuing discussion: (i) efforts to define cellular genes whose altered expression might contribute to tumour formation and (ii) efforts to decipher the mechanisms by which tumours are caused by RNA tumour viruses (retroviruses) lacking their own oncogenes.

II Identifying potential cellular genetic contributions to oncogenic mechanisms

At least two classes of host genes can now be considered candidates for some role in oncogenesis—a group of genes identified by their homology with retroviral oncogenes and another group identified by their capacity to induce transformation of a cultured mouse fibroblast cell line.

1 Cellular homologues of viral oncogenes

The first of these groups came into view during a search for the origins of the genetically defined transforming gene (*src*) of Rous sarcoma virus (RSV). Molecular hybridization reagents specific for *src* annealed to normal cellular DNA from the natural host for RSV (chickens) and to DNA from all other vertebrates, suggesting that the homologous cellular sequences had been relatively well conserved throughout evolution (Stehelin *et al.*, 1976; Spector *et al.*, 1978a). Tests for RNA and protein products of the *src*-related sequences demonstrated that they constituted a competent coding domain normally expressed at a low level (Spector *et al.*, 1978b; Collet *et al.*, 1978; Oppermann *et al.*, 1979). Additional tests for genetic linkages and polymorphisms, and for the structure of the domain, indicated that the *src*-related domain was a structurally conventional cellular gene (now called *c-src*), with multiple introns, little or no variation among members of a species, and no apparent linkage or resemblance to the endogenous proviruses that inhabit the germ lines of many animals (Hughes *et al.*, 1979a, b; Parker *et al.*, 1981; Shalloway *et al.*, 1981).

Subsequent studies of a large number of other RNA tumour viruses capable of transforming cultured cells and inducing tumours rapidly in animals have unearthed over fifteen distinct viral oncogenes, each of which is closely

related to (and presumably derived from) a cellular gene (Bishop, 1981; Bishop and Varmus, 1982). For convenient communication, these genes have been generically labelled '*v-onc*'s', the viral oncogenes, and '*c-onc*'s', their cellular counterparts (Coffin *et al.*, 1981). Each member of these classes has been assigned a trivial name (e.g., *src*, *myc*, *mos*; see Table 1) according to recently published rules. We may be approaching the limit of the number of *c-onc*'s that will ultimately be identified, since two related sets of oncogenes (*fes* and *fps, bas* and *ras*) have been isolated in viruses arising in different host species (Shibuya *et al.*, 1980; Andersen *et al.*, 1981); moreover, some oncogenes have appeared repeatedly in independent virus isolates within the same species (Table 1). Nevertheless, there may be additional cellular genes of similar potential that, for one reason or another, are not susceptible to transduction by retroviruses. Other means will be required to identify those genes.

An account of the functions and products of the several *c-onc*'s is beyond the purview of this short essay (see Bishop and Varmus, 1982). For our purposes, it is sufficient only to outline the evidence supporting the simple inference that at least some cellular homologues of *v-onc*'s do themselves have oncogenic potential. Such potential could be achieved in two ways: by mutations that alter the nature of the gene products or by modulations of gene expression that raise the level above a threshold required for neoplastic effects. For at least two *c-onc*'s (*c-mos* and *c-ras*), the latter possibility has been found sufficient by using a strong promoter of transcription to enhance expression of the cellular genes (Oskarsson *et al.*, 1980; Blair *et al.*, 1981;

Table 1. Cellular sequences identified as probable oncogenic sequences in the genomes of retroviruses

Onc sequence	*Number of virus isolates*	*Example*	*Animal origin*
src	>3	Rous sarcoma virus (Prague strain)	Chicken
fps	>3	Fujinimi sarcoma virus	Chicken
fes	2	Y73 sarcoma virus	Chicken
mos	1	UR-2 virus	Chicken
myc	4	Avian myelocytomatosis virus-29	Chicken
erb	1	Avian erythroblastosis virus	Chicken
myb	2	Avian myeloblastosis virus	Chicken
rel	1	Reticuloendotheliosis virus, strain T	Turkey
mos	2	Moloney murine sarcoma virus	Mouse
abl	1	Abelson murine leukaemia virus	Mouse
bas	1	BALB murine sarcoma virus	Mouse
ras	>3	Harvey murine sarcoma virus	Rat
fes	2	Snyder-Theilin feline sarcoma virus	Cat
fms	1	McDonough feline sarcoma virus	Cat
sis	1	Simian sarcoma virus	Woolly monkey

At least fifteen distinguishable sets of sequences (*v-onc*'s) have been found in the genomes of transforming retroviruses and shown to be homologous to host sequences (*c-onc*'s) with properties of cellular genes. Arbitrary names have been assigned to the *onc* sequences (Coffin *et al.*, 1981). The number of probably independent virus isolates containing each *onc* sequence, with an example of each, is listed with the host from which the *onc* sequences have apparently originated.

DeFeo *et al.*, 1981). These experiments required the molecular cloning and subsequent joining in vitro of two components: a cellular oncogene and a region of retroviral DNA, the long terminal repeat unit (LTR), which encodes a strong promoter. When reintroduced into cultured cells, the 'activated' *c-onc*'s are capable of transforming them to a neoplastic phenotype. Earlier experiments indicated that *c-src* could contribute at least partially to the production of an oncogenic protein, since competent transforming viruses could be isolated from tumours induced in chickens with deletion mutants of RSV lacking part of *v-src* (Hanafusa *et al.*, 1977). Biochemical studies of the recovered viruses suggested they were generated by recombination between *c-src* and the defective *v-src* (Wang *et al.*, 1979; Vigne *et al.*, 1980; for a dissenting view, see Lee *et al.*, 1981).

It should be emphasized, however, that for most *c-onc*'s there is as yet no direct evidence for their oncogenic potential; in these cases, the structural differences between *v-onc*'s and *c-onc*'s may prove to be critical determinants of function. Even in the provocative situations considered below, in which *c-onc*'s are activated in tumours induced by retroviruses that lack *v-onc*'s, it is premature to conclude that the enhanced expression of a *c-onc* is directly responsible for tumour growth.

2 Cellular genes that transform cultured cells

The second class of putative cellular tumour genes has been defined by using mouse NIH/3T3 fibroblasts to assay the transforming potential of naked DNA from a variety of sources, including chemically induced tumours, tumours induced by viruses lacking *onc* genes, spontaneous tumours, and normal tissues (Shih *et al.*, 1979, 1981; Cooper *et al.*, 1980; Cooper and Neiman, 1980; Lane *et al.*, 1981). Although the efficiency of transformation is quite low, DNA from the transformed cells can be used, in turn, to transform fresh cells; the transforming elements can be classified with respect to their susceptibility to restriction endonucleases or their linkage to highly repeated cellular sequences (Shih *et al.*, 1981; Murray *et al.*, 1981; Perucho *et al.*, 1981); and, in a few cases, the transforming components have been molecularly cloned in prokaryotic host-vector systems (Goldfarp *et al.*, 1982, G. Cooper, R. Weinberg, personal communications). Although little is presently known about the structures, products, and normal functions of these putative oncogenes, they are likely to be genetically altered in the tumour cells from which they are derived, since DNA similarly prepared from normal cells is inactive in the transformation assay. As in the case of *c-onc*'s and *v-onc*'s, it is for the most part uncertain whether the important differences reside in structural or in regulatory domains. The observation that the shearing of DNA from normal cells may confer transforming potential upon it suggests that regulatory changes may be more important, since the shearing might remove the restraining influence of *cis*-dominant controlling elements and allow potential transforming genes to be inserted within highly active transcriptional units (Cooper *et al.*, 1980). However, the experiments with normal cell DNA may be misleading: it is not known whether the transform-

ng activity of DNA from tumour cells is derived from the same set of genetic oci as the activity in sheared DNA from normal cells.

It is possible that transforming genes defined by the NIH/3T3 cell assay are on-overlapping with *c-onc*'s; however, there is reason (described below) for roposing that *c-onc*'s and transforming genes may play different roles in at east certain types of neoplasia. The number of potential transforming genes s still elusive, but it is striking that DNA from certain classes of tumour cells e.g., mammary tumours [Lane *et al.*, 1981], neural tumours and carcinomas Shih *et al.*, 1981], chemically transformed cell lines [Shilo and Weinberg 981], B or T cell leukaemias [Lane *et al.*, 1982] and human lung and bladder carcinomas [Perucho *et al.*, 1981; R. Weinberg, personal communiation]) exhibit class-specific patterns using restriction endonucleases. Moreover, the transforming element from two human colon and two human ung carcinomas appears to be the same (Perucho *et al.*, 1981; Murray *et al.*, 981). Such observations suggest that a common gene is affected in each umour type and that the number of potential transforming genes may be less han the number of distinguishable types of cancers.

Oncogenesis by viruses lacking *onc* genes

n the late 1970s, amidst the proliferation of viral oncogenes and their proucts, increasing attention was also given to the previously elusive question of ow some retroviruses can cause tumours despite the apparent absence of ansforming genes from the viral genomes. There were several reasons for is rekindled interest. First, the long latency between infection and the ppearance of tumours suggested that viruses lacking *v-onc*'s might be more evealing than *v-onc*$^+$ viruses about the pathogenesis of human tumours eich *et al.*, 1982). Second, the application of restriction mapping to proviruses tumours induced by *v-onc*$^-$ viruses indicated that these tumours were clonal r at least dominated by clones), so that the sites of proviral insertion could e conveniently studied (Steffen and Weinberg, 1978; Cohen *et al.*, 1979). hird, the newly perceived structure of proviral DNA—with identical gulatory domains (long terminal repeats or LTRs) present at both ends provoked hypotheses in which proviruses exerted regulatory influences ver flanking host DNA (Coffin, 1979; Quintrell *et al.*, 1980; Robinson *et al.*, 980).

For most tumours induced by viruses lacking oncogenes, pathogenetic echanisms are still far from understood. However, in one case, the induction B cell lymphomas in the bursa of Fabricius by avian leukosis viruses ALVs), there is strong evidence for insertion mutagenesis and gene activaon in the oncogenic process. The experimental findings that dominate the ory are as follows: (i) all tumours are clonal and carry at least a portion of an LV provirus (Neiman *et al.*, 1980; Payne *et al.*, 1981; Neel *et al.*, 1981; ung *et al.*, 1981); (ii) in several instances the provirus is structurally defecve and no viral genes are expressed, implying that viral gene products are ot necessary to maintain the tumour state (Payne *et al.*, 1981); (iii) virtually l tumours bear proviruses within the same cellular genetic domain (Neel *et*

al., 1981; Payne *et al.*, 1981), a region first identified by Hayward and his colleagues (1981) as the *c-onc* called *c-myc*; (iv) all the tumours bearing insertions in the *c-myc* region exhibit levels of expression of *c-myc* RNA 10- to 100-fold above levels in normal B cells (Hayward *et al.*, 1981; Payne *et al.*, 1982); and (v) DNA from the tumours contains activated transforming genes, as determined by assay in NIH/3T3 cells, with the transforming DNA free of ALV or *c-myc* sequences (Cooper and Neiman, 1980, 1981).

How should these findings be viewed in relation to the pathogenesis of the disease? It is likely that ALV spreads efficiently through the bursal cell population in a newly infected susceptible animal, inserting proviral DNA more or less at random in the genomes of large numbers of cells. In the rare cell (about 1 in 10^6) that acquires a provirus in the *c-myc* domain, the neoplastic process can presumably be initiated by overproduction of *c-myc* RNA and protein. (The *c-myc* protein has not been identified, and there is no proof that it is inherently oncogenic; furthermore, the *v-myc* domain is usually expressed in virally transformed cells as a hybrid protein also containing peptides encoded by viral structural genes [Bister *et al.*, 1977].) It is probable that additional mutations or rearrangements of cellular genes occur subsequent to activation of *c-myc*, including one change that activates a gene capable of transforming NIH/3T3 cells. As the tumour nodule enlarges and the genetically evolving cellular population competes for dominance of the tumour mass, advantage belongs to tumour cells in which all proviruses, including the inciting provirus in the *c-myc* domain, are inactivated (e.g., by partial deletions), since any immune response against viral antigens will no longer operate against such cells. However, retention of at least a single LTR near *c-myc* may be necessary to maintain the tumour state, even though viral gene products are not required (Payne *et al.*, 1981).

An immediate question raised by the study of chicken lymphomas is relevant to understanding gene regulation in eukaryotes, as well as tumorigenesis: how does an insertion of ALV proviral DNA amplify the expression of *c-myc*? The initial premise, and the simplest, was that in each case an ALV LTR would be positioned 'upstream' from the genetic target, in an orientation that would permit it to act as a promoter for transcription (Neel *et al.*, 1981; Payne *et al.*, 1981). Although the majority of tumours do contain this arrangement of *c-myc* and an ALV LTR, with RNA transcripts that appear to have been initiated within the LTR and extended into *c-myc* (Hayward *et al.*, 1981), there is also a substantial number of tumours in which other arrangements are observed (Payne *et al.*, 1982). In several tumours, the ALV provirus is upstream from *c-myc*, but in the transcriptional orientation opposite that of *c-myc*; in at least one tumour the provirus is downstream from *c-myc*. With these two unexpected arrangements, the ALV LTR cannot act simply as a promoter to enhance the expression of *c-myc*. The most obvious possibility is that the ALV LTRs indirectly potentiate the transcriptional activity of the chromosomal domain in which they have been inserted, affecting the strength of promoters normally used or recruiting promoters normally inactive.

Although the biochemical bases for such phenomena are not understood,

potentially related findings in more malleable contexts suggest that the phenomena are experimentally accessible. Thus, regions near the origin of replication in SV40 DNA and the LTR of RSV (but not the LTR of the non-tumorigenic chicken virus, RAV-0) markedly increase the frequency of transformation of thymidine kinase-deficient (tk^-) mouse cells to a tk^+ phenotype when present in a micro-injected plasmid containing the complete herpes simplex *tk* gene in either orientation (Capecchi, 1980; P. Luciw and M. Capecchi, unpublished). Enhancing properties of SV40 DNA upon the expression of linked promoters have been mapped within a 72 bp repeat normally positioned upstream from the promoter for the early genes; this sequence has a strong influence upon transcription of the early genes (Gruss *et al.*, 1981; Benoist and Chambon, 1981); it can stimulate expression of linked heterologous genes using their natural promoters or adjacent promoter-like sequences (Banerji *et al.*, 1981; M. Fromn and P. Berg, personal communication; M. Botchan, personal communication); and it can be replaced during SV40 replication by a functionally homologous domain of the murine sarcoma virus LTR (Levinson *et al.*, 1982).

The findings with ALV-induced lymphomas raise a host of additional questions, the most approachable being whether similar events occur in tumours induced by other viruses without *onc* genes. Several pieces of evidence encourage belief that they do. (i) In avian B cell lymphomas induced by a retrovirus unrelated to ALV (the reticuloendotheliosis virus called chicken syncytial virus) and by a virus differing only modestly from ALV (the myeloblastosis-associated virus, MAV), proviral insertions near *c-myc* have been identified, sometimes accompanied by deletions within and amplification of the interrupted domain (Noori-Daloii *et al.*, 1981; D. Westaway and C. Moscovici, unpublished). However, the level of *c-myc* expression has yet to be determined in these cases and tumour DNA has not been tested for transforming activity in NIH/3T3 cells. (ii) Preliminary evidence implicates proviral insertion near another cellular oncogene (*c-erb*), the progenitor of the transforming gene of avian erythroblastosis virus, in the rare induction of erythroblastosis by ALV (T. Fung and H. J. Kung, personal communication). (iii) A significant proportion (about 50%) of mammary carcinomas induced by the mouse mammary tumour virus (MMTV) in C3H mice contain proviruses in the same 20 kb region of the host genome (R. Nusse, unpublished). However, this region has been defined only by the presence of proviral DNA: no known cellular oncogenes reside within it, and an activated transcriptional unit has not been found. The analogy with ALV-induced lymphomas is strengthened by the observation that DNA from MMTV-induced mammary tumours transforms NIH/3T3 cells; the pattern of inactivation of the transforming principle by restriction endonucleases suggests that the same oncogene may be involved in each of five cases and in a chemically induced mouse mammary tumour and a spontaneous human mammary carcinoma (Lane *et al.*, 1981). As in the case of ALV-induced lymphomas, the transforming sequences seem to be unlinked to proviral DNA.

It would be premature to conclude from these provisional data that all retroviruses without oncogenes induce tumours in similar ways. On the one

hand, we are far from understanding how oncogenic transformation occurs in the most successfully explored example, ALV-induced lymphoma; on the other hand, some of the most important viruses in this category—including murine, bovine, and feline leukaemia viruses, as well as the newly described human isolates associated with T cell lymphomas and leukaemias (Poiesz *et al.*, 1980; Hinuma *et al.*, 1981)—have yet to be adequately assessed from these new perspectives.

Some insight into the significance and function of putative oncogenes affected in various tumours may be provided by asking whether viruses, such as ALV, that induce several kinds of neoplasm do so by activating the same or different oncogenes in each target cell. Conversely, it will be of interest to determine whether the same oncogene is affected by different viruses in a single target cell, as may be the case for *c-myc* in B cell lymphomas (see above). It is possible that the answers to these questions will depend upon the normal functions of the various cellular oncogenes (e.g., whether they are general regulators of growth or determinants of differentiation); in any case, the answers may serve as guides for exploring the involvement of such genes in spontaneous tumours and in tumours induced by non-viral agents.

A profound understanding of neoplastic mechanisms involving putative cellular oncogenes will doubtless demand detailed descriptions of the products of these genes. There is reason to be optimistic about achieving these immediate goals, judging from recent successes with viral oncogenes (reviewed in Tooze, 1980 and Bishop and Varmus, 1982). It will be a more difficult task to assign functional attributes to the genes and their products. The demanding question that now dominates the study of viral oncogenes will ultimately need to be addressed with cellular oncogenes as well: What biochemical properties of their products are responsible for the various manifestations of the neoplastic phenotype?

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